Automated large-scale purification of a G protein-coupled receptor for neurotensin

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Abstract Structure determination of integral membrane proteins requires milligram amounts of purified, functional protein on a regular basis. Here, we describe a protocol for the purification of a G protein-coupled neurotensin receptor fusion protein at the 3-mg or 10-mg level using immobilized metal affinity chromatography and a neurotensin column in a fully automated mode. Fermentation at a 200-l scale of *Escherichia coli* expressing functional receptors provides the material needed to feed into the purification routine. Constructs with tobacco etch virus protease recognition sites at either end of the receptor allow the isolation of neurotensin receptor devoid of its fusion partners. The presented expression and purification procedures are simple and robust, and provide the basis for crystallization experiments of receptors on a routine basis.

Key words: Large-scale purification; G protein-coupled receptor; Automation; Detergent; Neurotensin receptor

1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins involved in important physiological processes, including cell-to-cell communication, mediation of hormonal activity and sensory transduction (for reviews see [1,2]). About 1000 GPCRs have been identified, 300–400 of which are found throughout the body binding endogenous ligands (the remainder are chemosensory GPCRs for odors, pheromones or taste) [3]. Many of them have been implicated as major therapeutic routes to the treatment of human diseases. Despite the striking clinical relevance of GPCRs, only one high-resolution structure (rhodopsin) is known [4,5]. One contributing factor to the structure determination of rhodopsin was its availability in large quantities from natural sources. In contrast, recombinant overexpression followed by efficient purification methods are needed for GPCRs that occur naturally at low levels [6] (see also *Biochim. Biophys. Acta*, vol. 1610, special issue on Overexpression of Integral Membrane Proteins).

Neurotensin (NT) is a 13-amino acid peptide that is involved in a variety of neuromodulatory functions in the central and peripheral nervous system [7,8]. NT binds to its receptors NTS1 and NTS2 [9–12]. The levocabastine-insensitive neurotensin type I receptor (NTS1) [10–12] interacts with the agonist NT with high affinity. Similar observations were made for the N-terminally truncated form of rat NTS1 when expressed as a maltose-binding protein (MBP) fusion in *Escherichia coli* [13,14] and purified in the presence of detergents [14,15].

Crystallization and other applications such as nuclear magnetic resonance (see [16,17,28,29]) require milligram quantities of functional GPCRs. Only a few receptors have been purified in such quantities (see [18–22,30]). Large-scale purification schemes must be simple and robust to give reproducible results on a regular basis. Here, we report the purification of a G protein-coupled neurotensin receptor fusion protein at the 3-mg or 10-mg level using two successive affinity columns in a fully automated mode.

2. Materials and methods

2.1. Expression of NTS1 fusion proteins in shake flasks

The NTS1 fusion protein MBP-rT43NTR-TrxA-H10 (NTS1–624) consists of the *E. coli* MBP (Lys[1] to Thr[366]), followed by Gly-Ser, the N-terminally truncated rat NT type I receptor NTS1 (rT43NTR, Thr[43] to Tyr[424]) [11], three Ala residues, the *E. coli* thioredoxin (TrxA, Ser[2] to Ala[109]), Gly-Thr and a decahistidine tag (H10) [23]. The fusion protein MBP-N10-Tev-rT43NTR-N5G3S-Tev-G3S-TrxA-H10 (NTS1–1023) contains two tobacco etch virus (Tev) protease recognition sites [24,25] surrounding rT43NTR, with Gly-Ser-Asn_{10}-Glu-Asn-Leu-Tyr-Phe-Gln-Ser-Gly-Ser between MBP and rT43NTR, and Ala_3-Asn_5-Gly_3-Ser-Glu-Asn-Leu-Tyr-Phe-Gln-Ser-Gly_3-Ser-Glu-Phe between rT43NTR and TrxA-H10 [17]. In the fusion protein MBP-N10-Tev-rT43NTR-CH2-N5G3S-G3S-TrxA-H10 (NTS1–1233), the C-terminus of rT43NTR is changed from Glu[421]-Thr-Leu-Tyr[424] to Glu[421]-Asn-Leu-Tyr-Phe-Gln-Ser (to constitute a Tev protease recognition site), followed by Asn_5-Gly_3-Ser-Gly_3-Ser-Glu-Phe and TrxA-H10. Derivatives of the expression vector pRG/III-hs-MBP [14] were used for the production of functional, membrane-inserted receptors in *E. coli* DH5α. Cells were grown in 750 ml of double-strength TY medium (BIO 101 systems Q-Biogene) containing ampi-

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHS, cholesteryl hemisuccinate Tris salt; GPCR, G protein-coupled receptor; H10, decahistidine tag; LM, *n*-dodecyl-β-D-maltoside; MBP, *E. coli* maltose-binding protein; NTS1–624, fusion protein MBP-rT43NTR-TrxA-H10 (see Section 2.1); NTS1–1023, fusion protein MBP-N10-Tev-rT43NTR-N5G3S-Tev-G3S-TrxA-H10; NTS1–1233, fusion protein MBP-N10-Tev-rT43NTR-CH2-N5G3S-G3S-TrxA-H10; rT43NTR, N-terminally truncated rat neurotensin type I receptor NTS1; Tev, tobacco etch virus; TrxA, *E. coli* thioredoxin

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cillin (67 μg/ml) and glucose (0.2% w/v) in baffled 2-l flasks at 37°C to an OD_{600} of 0.5. After induction with 0.25 mM isopropyl-β-thiogalactoside (IPTG), the temperature was decreased to 22°C. The cells were harvested about 40 h later, frozen in liquid nitrogen, and stored at −85°C [14].

2.2. Large-scale fermentation

Two hundred liters of double-strength TY medium (Difco) containing ampicillin and glucose (see above) in a 300-l working volume fermenter (New Brunswick Scientific) were inoculated with 200 ml of an overnight culture grown at 37°C. Cells were grown at 37°C to an OD_{600} of 0.5 with constant stirring (200 rpm) and air supply of 1 vvm (volume per volume per minute). After induction with 0.25 mM IPTG, the temperature was decreased to 22°C. The cells were harvested about 40 h later using a continuous flow centrifuge (Sharples As 16P) at a flow rate of 100 l/h, frozen in liquid nitrogen, and stored at −85°C.

2.3. Automated large-scale purification of NTS1 fusion proteins

2.3.1. Reagents and buffers. Cholesteryl hemisuccinate Tris salt (CHS), and the detergents *n*-dodecyl-β-D-maltoside (LM) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from Anatrace (Maumee, OH, USA). Stock solutions (6% CHAPS/0.12% CHS and 10% LM) were made fresh prior to each purification. Protease inhibitor stock solutions were made to 70 mg/ml (phenylmethylsulfonyl fluoride), 1 mg/ml (leupeptin) and 1.4 mg/ml (pepstatin A). The following buffers were used [15]: 2×solubilization buffer: 100 mM Tris–HCl pH 7.4, 60% glycerol, 400 mM NaCl; NiA buffer: 50 mM Tris–HCl pH 7.4, 30% glycerol, 50 mM imidazole, 200 mM NaCl, 0.5% CHAPS/0.1% CHS, 0.1% LM; NiB buffer: NiA with 200 mM imidazole; NT0 buffer: 50 mM Tris–HCl pH 7.4, 30% glycerol, 1 mM EDTA, 0.5% CHAPS/0.1% CHS, 0.1% LM; NT200 buffer: NT0 with 200 mM NaCl; NT1K buffer: NT0 with 1 M NaCl; A3 buffer: 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 M NaCl; NT column storage buffer B3: 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 3 mM NaN_3.

2.3.2. Solubilization. One hundred grams (250 g) of cells were homogenized in 250 ml (500 ml) of 2×solubilization buffer using a Waring blender for 3–5 min. The following steps were carried out at 4°C or on ice. After addition of 250 μl (500 μl) of each protease inhibitor, 2.5 ml (5 ml) of a 1 M $MgCl_2$ solution and 200 μl (300 μl) of DNase I stock (Sigma D-4527, 10 mg/ml), 50 ml (100 ml) of each detergent stock solution were added dropwise with gentle stirring. The mixture was sonicated for 13 min (33 min) (Misonix sonicator 3000, 1/2 inch flat tip, level 4, 1 s on, 2 s off). The volume was then adjusted to 500 ml (1 l) with H_2O. 250 μl (500 μl) of each protease inhibitor were added, and the mixture was stirred for a further 30 min. Cell debris was removed by centrifugation (Beckman 45 Ti rotor, 45 000 rpm, 1 h). Imidazole (2 M stock solution, adjusted to pH 7.4) was added to the supernatant to give a final concentration of 50 mM [23]. The sample was passed through a 0.2-μm filter (Stericup, Millipore) and then used for purification by immobilized metal affinity chromatography.

2.3.3. Automated purification of NTS1 fusion proteins. A Purifier P100 system (Amersham Biosciences) was equipped with a sample valve (buffer line S1 with air sensor Air-912 for sample loading, and S8 for rinsing the sample pump with buffer NiA), sample pump P950, injection valve (with the sample pump outlet connected to port 2, and port 3 connected to port 6), column valves, outlet valve, fraction collector Frac950, buffer valve A1 (with buffer lines A11 for H_2O, A12 for buffer NT0, A13 for buffer NT1K, A16 for buffer B3, A17 for buffer A3, A18 for 20% ethanol) connected to buffer line A1, buffer line A2 for NiA buffer, buffer line B1 for NiB buffer, and buffer valve B2 (with buffer lines B21 for H_2O, B22 with air sensor Air-912 for loading of the Ni-NTA eluate onto the NT column, B27 for 20% ethanol, B28 for NT200 buffer) connected to buffer line B2 (Fig. 1). Teflon tubing (i.d. 1.6 mm) was used as buffer inlet lines, whereas Tefzel tubing (i.d. 0.75 mm) was used as the air sensor inlet lines. A 50-ml or 100-ml Ni-NTA Superflow (Qiagen) column (XK50, Amersham Biosciences) and a 10-ml or 20-ml NT column [14,15] (XK26) were used for the purification of NTS1 fusion proteins at the 3-mg or 10-mg level, respectively. The following details refer to a 10-mg purification. Briefly, solubilized receptors (∼960 ml) were passed through line S1 by an external sample pump onto the Ni-NTA column at a flow rate of 2 ml/min. An air sensor detected the end of loading. After extensive washing of the Ni-NTA column with 1500 ml of buffer NiA using system pump A, bound receptor fusion protein was eluted in the presence of 200 mM imidazole (system pump B) at 2 ml/min, and collected (line F3) into a vessel which contained 1 ml of buffer NT200, into which line B22 was submersed. To reduce the NaCl and imidazole concentration from 200 mM to 70 mM to allow binding of NTS1 to the NT column, the Ni-NTA column eluate (line B22, 35% B) was diluted with NT0 buffer (line A12) using the system pumps, and passed onto the NT column at 0.4 ml/min. Again, an air sensor detected the end of loading. After washing of the NT column, NTS1 fusion proteins were eluted with NT1K buffer and collected. The step-by-step protocols for the automated column equilibrations and purification of NTS1 fusion proteins at the 3-mg or 10-mg level are available upon request.

2.4. Processing of NTS1 fusion proteins by Tev protease

2.4.1. Expression and purification of Tev protease. Intracellular expression as MBP fusion and in vivo autoprocessing of the Tev protease mutant S219V was done as described using the strain BL21-RIL pRK793 [26]. Cells were broken with a French press (1 passage at 1200 psi), and the histidine-tagged Tev protease was purified by Ni-NTA chromatography (column buffer A: 50 mM NaP_i pH 8.0, 100 mM NaCl, 10% glycerol, 25 mM imidazole; elution buffer B: buffer A with 200 mM imidazole). The Ni-NTA column eluate containing the His-Tev(S219V)-Arg protease was pooled, diluted 1:1 with glycerol, frozen in liquid nitrogen, and stored at −20°C. The protein concentration was 1 mg/ml.

2.4.2. Cleavage of NTS1 fusion proteins by Tev protease. To determine the amount of His-Tev(S219V)-Arg protease necessary for complete processing of NTS1 fusion proteins, receptors (NT column eluate, in NT1K buffer) were incubated at 4°C for ∼15 h with increasing concentrations of Tev protease in the presence of 1 mM dithiothreitol (data not shown). Complete cleavage of NTS1 fusion proteins was obtained at a ratio of 1:1 (mol/mol).

2.4.3. Purification of rT43NTR by gel filtration. Purified fusion protein NTS1–1233 (5.4 mg) was incubated with an equimolar amount of Tev protease in a total of 8 ml at 4°C overnight. The mixture was concentrated in a Centriprep-50 device (Millipore) to 3 ml, and applied to a HiLoad 16/60 Superdex 200 prep grade column (Amersham Biosciences) equilibrated with GF buffer (50 mM Tris–HCl pH 7.4, 15% glycerol, 1 mM EDTA, 1 M NaCl, 0.5% CHAPS/0.1% CHS, 0.1% LM). Proteins were separated at a flow rate of 0.4 ml/min, and 1-ml fractions were collected.

2.5. Protein and ligand-binding analysis

The protein content was measured according to the method of Schaffner and Weissmann [27] with bovine serum albumin as the standard. Specific agonist binding was determined at a [^{3}H]NT (Perkin-Elmer) concentration of 2 nM in assay buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.1% bovine serum albumin, 0.004% bacitracin). Ligand-binding analysis with intact cells [13,14] was carried out in a volume of 0.5 ml for 4 h on ice. Separation of bound from free ligand was achieved by rapid filtration through GF/B glass fiber filters (Whatman) pretreated with polyethyleneimine. Ligand-binding analysis with detergent-solubilized receptors was done in assay buffer containing detergent (0.1% LM, 0.2% CHAPS, 0.04% CHS) [14,15] for 1 h on ice. Separation of receptor–ligand complex from free ligand was achieved by centrifugation-assisted gel filtration using Bio-Spin 30 Tris columns (Bio-Rad) according to the manufacturer's instructions. Samples were analyzed by liquid scintillation counting.

3. Results and discussion

3.1. Large-scale fermentation

To provide the starting material for automated purification of NTS1 fusion proteins on a regular basis, fermentation at a 200-l scale was carried out. [^{3}H]NT ligand-binding analyses with intact cells gave ∼1000 receptors/cell, a value comparable to that obtained in shake flask experiments [13–15]. The cell density at harvest was 2.5–3 OD_{600}. One 200-l run yielded about 1.1 kg of wet bacterial cell paste with 90 mg of functional receptor fusion protein (data are averaged from five fermenter runs). Attempts to increase the biomass at harvest,

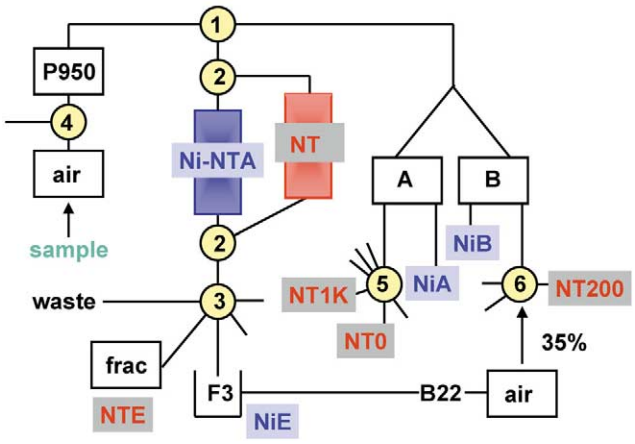

Fig. 1. Schematic representation of the chromatography system used for automated purification of NTS1 fusion proteins. Circles denote valves (1, injection valve; 2, column valves; 3, outlet valve; 4, sample valve; 5, buffer valve A1; 6, buffer valve B2). The system pumps are indicated as A and B. Buffer lines F3 and B22 are used to collect and to load the Ni-NTA column eluate (NiE) onto the NT column. The NT column eluate (NTE) is collected by the fraction collector. The composition of the buffers NiA, NiB, NT0, NT200 and NT1K are given in Section 2. For clarity, not all buffers and buffer lines are shown.

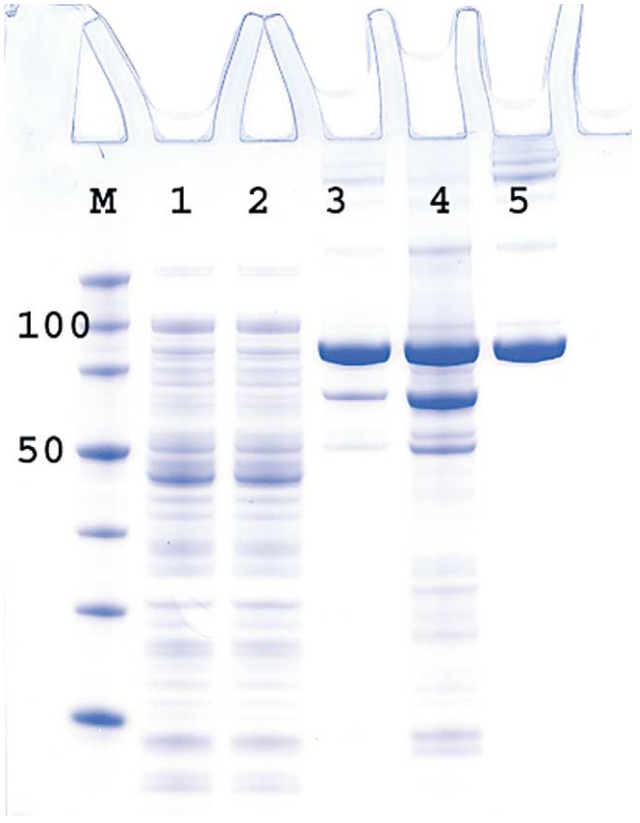

Fig. 2. Automated 10-mg preparation of the fusion protein NTS1–624. The NTS1 fusion protein was purified on a 100-ml Ni-NTA column followed by a 20-ml NT column, starting from 250 g of cells. The progress of purification was monitored by SDS–PAGE (NuPAGE 4–12% Bis-Tris gel, Invitrogen, 1×MES buffer) and Coomassie R-250 staining. Lane M: Novagen Perfect Protein Marker (15–150 kDa); lane 1: 10 μg of supernatant; lane 2: 10 μg of Ni-NTA column flowthrough; lane 3: 5 μg of Ni-NTA column eluate; lane 4: 10 μg of NT column flowthrough; lane 5: 5 μg of NT column eluate.

by inducing expression at a cell density higher than OD_{600} of 0.5 or by using richer media, resulted in lower levels of functional receptors per cell. The best results in expression and purification of NTS1 fusion proteins were obtained by mimicking the growth conditions in shake flasks.

3.2. Automated purification

The preparation of GPCRs in functional form must be reproducible to provide high-quality material for regular crystallization experiments. A 'manual', two-step purification procedure, immobilized metal affinity chromatography followed by a NT column, has been shown to give functional, pure NTS1 fusion protein [15] at small scale, and we have adapted this procedure to the production of receptors in milligram quantities [17]. However, the increased volumes of sample and column buffers, combined with slow flow rates needed

Table 1
Automated large-scale purification of the fusion protein NTS1–624

	Volume (ml)	Functional receptor (nmol)	Protein (mg)	Specific binding (nmol/mg)	Purification (fold)	Recovery (%)
100 g of cells, 50-ml Ni-NTA column, 10-ml NT column						
supernatant	478	82.8	5214	0.016	1	100
NiFT	548	11.8	ND	ND	NA	14
NiE	60	ND	ND	ND	ND	
NTFT	207	ND	4.9	ND	NA	
NTE	8	32.6	3.2	10.2	640	39
250 g of cells, 100-ml Ni-NTA column, 20-ml NT column						
supernatant	934	193.5	13562	0.014	1	100
NiFT	1070	22.4	ND	ND	NA	12
NiE	122	145.5	30	4.8	345	75 (100)
NTFT	412	4.3	13	ND	NA	2 (3)
NTE	14	103.8	9.6	10.8	774	53 (71)

Data for the 3-mg preparation are the average of four purifications. Data for the 10-mg preparation are taken from one representative experiment. NiFT contains the actual flowthrough and buffer used to wash the sample pump. The volume of the NTFT is larger than that of the NiE because of the dilution applied to reduce the NaCl and imidazole concentrations. Recovery data listed in parentheses refer to the NT column. A theoretical value for specific binding of 10.4 nmol/mg is calculated for NTS1–624 (molecular mass of 96.5 kDa) assuming one ligand-binding site per receptor molecule. Abbreviations: NA, not applicable; ND, not determined; NiE, Ni-NTA column eluate; NiFT, flowthrough of Ni-NTA column; NTE, NT column eluate; NTFT, flowthrough of NT column.

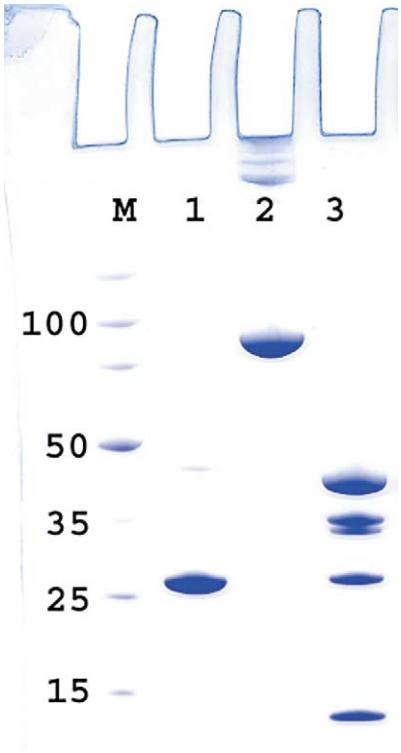

Fig. 3. Identification of a cryptic Tev protease recognition site at the C-terminus of NTS1. The fusion protein NTS1–1023 was purified by immobilized metal affinity chromatography and a NT column, and incubated with Tev protease. The products were analyzed by SDS–PAGE (NuPAGE 4–12% Bis-Tris gel, Invitrogen, 1×MES buffer) and Coomassie R-250 staining. Lane M: Novagen Perfect Protein Marker (15–150 kDa); lane 1: 6.6 μg of Tev protease (calculated MW = 28.6 kDa); lane 2: 6.7 μg of purified NTS1–1023 (calculated MW = 100.9 kDa); lane 3: 12.5 μg of the protein mixture after incubation with Tev protease (calculated MW of MBP = 42.3 kDa, calculated MW of rT43NTR = 44.8 kDa, calculated MW of TrxA-H10 = 13.8 kDa). N-terminal sequence analysis and in-gel tryptic digest followed by LC/MS/MS identified two receptor species with identical N-termini but different C-termini and an apparent MW smaller than calculated.

to obtain high receptor yields, made this procedure impractical. Therefore, automation of sample loading and successive processing of the two affinity columns was desirable [18].

Fig. 1 shows the chromatography scheme for the automated purification of NTS1 fusion proteins. The program routines ensure that each buffer line, pump head and column are filled with the correct buffer at any given time. An air sensor detects the end of sample loading, and initiates the Ni-NTA column wash. The apparent capacity of the Ni-NTA resin in our buffer system is low (< 0.5 mg of NTS1 fusion protein/ml resin, see Table 1), hence the use of large columns. The Ni-NTA column eluate is collected in a vessel connected to a second air sensor for loading of the NT column. However, the apparent affinity of NT for NTS1 is reduced in the presence of the high concentrations of sodium ions and imidazole in the NiB buffer [15] precluding binding of functional receptors to the NT column. The necessary change in buffer composition is accomplished by diluting the Ni-NTA column eluate with NT0 buffer just prior to loading of the NT column, using the system pumps. After elution of receptors from the NT column and collection, the columns are washed into storage buffer, and the system pumps are cleaned with H_2O and 20% ethanol. Starting with solubilization, a 3-mg receptor preparation is available after 31 h, whereas a 10-mg preparation is completed after 55 h. Fig. 2 and Table 1 detail the large-scale purification of the fully functional receptor fusion protein NTS1–624. Similar results at the 3-mg and 10-mg level have been obtained for NTS1–1023 and NTS1–1233 (data not shown).

3.3. Generation of rT43NTR

3.3.1. Identification of a cryptic Tev protease recognition site at the C-terminus of NTS1. The purified fusion protein NTS1–1023 with Tev protease recognition sites at either end of rT43NTR was subjected to protease treatment, and the proteolytic fragments were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 3). rT43NTR showed an apparent MW smaller than the calculated MW of 44.8 kDa. Unexpectedly, rT43NTR was found

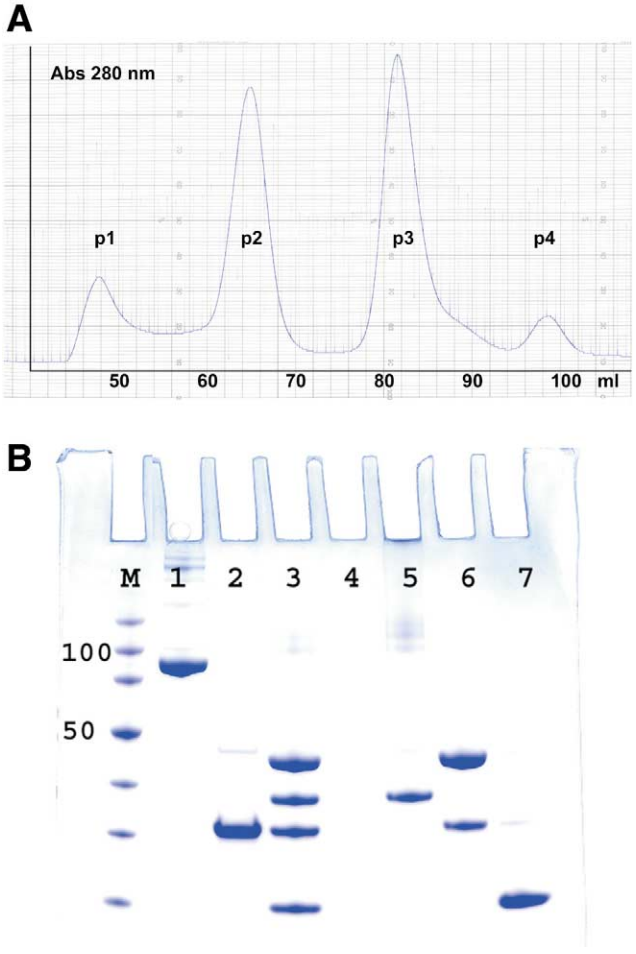

Fig. 4. Purification of rT43NTR. NTS1–1233 was purified by immobilized metal affinity chromatography and a NT column, and incubated with Tev protease. The resulting proteins were separated by gel filtration (A) and analyzed by SDS–PAGE (B) (NuPAGE 4–12% Bis-Tris gel, Invitrogen, 1×MES buffer) and Coomassie R-250 staining. Lane M: Novagen Perfect Protein Marker (15–150 kDa); lane 1: 5 μg of purified NTS1–1233; lane 2: 5 μg of Tev protease; lane 3: 12 μg of the protein mixture after incubation with Tev protease; lane 4: gel filtration peak 1; lane 5: gel filtration peak 2, 5 μg of rT43NTR (calculated MW = 43.3 kDa); lane 6: gel filtration peak 3, 5 μg of protein (MBP, Tev protease); lane 7: gel filtration peak 4, 5 μg of TrxA-H10. Note that gel filtration peak 1 contains hardly any protein.

as two species. N-terminal sequence analysis and in-gel tryptic digest followed by LC/MS/MS identified identical N-termini for the two proteins. However, two different putative C-terminal tryptic peptides were found (Glu-Thr-Leu-Tyr-Ala_3-Asn_5-Gly_3-Ser-Glu-Asn-Leu-Tyr-Phe-Gln and Glu-Thr-Leu-Tyr-Ala-Ala, data not shown). The canonical Tev protease recognition site is given as Glu-Asn-Leu-Tyr-Phe-Gln↓Gly/Ser [24]. Three amino acid residues of the NTS1 C-terminus (Glu, Leu, Tyr) are identical to the canonical Tev protease recognition site. This receptor region is recognized by Tev protease, although less efficiently (the smaller receptor fragment is less abundant, see Fig. 3).

3.3.2. Preparation of rT43NTR. To obtain a uniform receptor species, the sequence of the rT43NTR C-terminus was changed from Glu^{421}-Thr-Leu-Tyr^{424} to Glu^{421}-Asn-Leu-Tyr-Phe-Gln-Ser to constitute a canonical Tev protease recognition site. The correct processing by Tev protease of NTS1–1233 was confirmed by in-gel tryptic digest and mass spectrometry (data not shown) with the receptor C-terminus ending in Glu^{421}-Asn-Leu-Tyr-Phe-Gln. The receptor was separated from MBP, Tev protease and TrxA-H10 by gel filtration (Fig. 4) with a yield of 1.6 mg rT43NTR (69% of theoretical recovery) starting from 5.4 mg of fusion protein. Similar recovery yields (70–80%) were obtained in three further experiments (data not shown). Ligand-binding analysis gave a B_{max} value of 19.7 nmol/mg (a theoretical value for specific binding of 23.1 nmol/mg is calculated for rT43NTR (molecular mass of 43.3 kDa) assuming one ligand-binding site per receptor molecule) (data presented are from one representative experiment). Careful inspection of the rT43NTR preparation showed some minor contaminants originating from the Tev protease preparation, which cannot easily be seen in Fig. 4. Future optimization of the gel filtration step is required to remove these unwanted proteins.

4. Conclusions

The presented large-scale purification of NTS1 fusion proteins is simple and robust. It reliably provides high-quality receptor material for crystallization and other applications such as antibody generation. Receptors are solubilized from whole cells as starting material, rather than crude membranes, which are time-consuming to prepare. The use of a H10 tag allows the efficient enrichment of receptors by immobilized metal affinity chromatography. The subsequent NT column selects for functional protein. The use of air sensors and automated column processing minimizes the time needed for purification.

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